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Molecular Cloning, Sequencing, and Heterologous Expression of the *vao*A Gene from *Penicillium simplicissimum* CBS 170.90 Encoding Vanillyl-Alcohol Oxidase*

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The cDNA encoding vanillyl-alcohol oxidase (EC 1.1.3.7) was selected from a cDNA library constructed from mRNA isolated from *Penicillium simplicissimum* CBS 170.90 grown on veratryl alcohol by immunochemical screening. The *vao*A-cDNA nucleotide sequence revealed an open reading frame of 1680 base pairs encoding a 560-amino acid protein with a deduced mass of 62,915 Da excluding the covalently bound FAD. The deduced primary structure shares 31% sequence identity with the 8α-(*O*-tyrosyl)-FAD containing subunit of the bacterial flavocytochrome *p*-cresol methyl hydroxylase.

The *vao*A gene was isolated from a *P. simplicissimum* genomic library constructed in λ_{EMBL3} using the *vao*A-cDNA as a probe. Comparison of the nucleotide sequence of the *vao*A gene with the cDNA nucleotide sequence demonstrated that the gene is interrupted by five short introns.

***Aspergillus niger* NW156 *prt*F *pyr*A *leu*A *csp*A transformed with the *pyr*A containing plasmid and a plasmid harboring the complete *vao*A gene including the promoter and terminator was able to produce *vao*A mRNA and active vanillyl-alcohol oxidase when grown on veratryl alcohol and anisyl alcohol. A similar induction of the *vao*A gene was found for *P. simplicissimum*, indicating that similar regulatory systems are involved in the induction of the *vao*A gene in these fungi.**

Introduction of a consensus ribosome binding site, AGAAGGAG, in the *vao*A-cDNA resulted in elevated expression levels of active vanillyl-alcohol oxidase from the *lac* promoter in *Escherichia coli* TG2. The catalytic and spectral properties of the purified recombinant enzyme were indistinguishable from the native enzyme.

Vanillyl-alcohol oxidase (EC 1.1.3.7) is a novel type of flavoprotein oxidase that was first isolated from *Penicillium simplicissimum* CBS 170.90 grown on veratryl alcohol (1). The enzyme is a homo-octamer with each 65-kDa subunit harboring an 8α-(N^3-histidyl)-FAD (2). Vanillyl-alcohol oxidase has a broad substrate specificity. In addition to the conversion of vanillyl alcohol to vanillin (Equation 1), the enzyme catalyzes the conversion of a wide range of phenolic compounds bearing side chains of variable size at the para-position of the aromatic ring (3, 4). Due to its broad substrate spectrum, vanillyl-alcohol oxidase may be applied in the fine chemical industry (5). Based on induction experiments, 4-(methoxymethyl)phenol has been proposed to represent the physiological substrate (6). Recently, from rapid reaction kinetics conclusive spectral evidence was obtained that the vanillyl-alcohol oxidase-mediated oxidative demethylation of 4-(methoxymethyl)phenol proceeds through the formation of a quinone-methide product intermediate (4). In the absence of oxygen, this intermediate is stabilized in the active site of the reduced enzyme. Upon flavin reoxidation, the quinone methide of 4-(methoxymethyl)phenol readily reacts with water, yielding 4-hydroxybenzaldehyde, methanol, and hydrogen peroxide as final products.

Recently, the three-dimensional structure of vanillyl-alcohol oxidase was elucidated (7). The crystallographic analysis corroborated earlier observations (2, 8) that the vanillyl-alcohol oxidase octamer can be described as a tetramer of tightly interacting dimers. Each vanillyl-alcohol oxidase monomer consists of two domains. The larger domain creates a binding site for the ADP moiety of the FAD, whereas the smaller cap domain covers the active site that is located between the two domains. Furthermore, from the structures of several vanillyl-alcohol oxidase inhibitor complexes, it could be deduced that the shape of the active site cavity controls substrate specificity (7).

In this paper we describe the cloning, sequencing, and expression of the gene encoding vanillyl-alcohol oxidase from *P. simplicissimum* CBS 170.90, providing the necessary amino acid sequence information which together with the three-dimensional structure establishes the basis for future protein engineering studies.

EXPERIMENTAL PROCEDURES

Strains and Media—Growth conditions for *P. simplicissimum* (CBS 170.90; ATCC 90172) have been described before (6). *Aspergillus niger* NW156 (*prt*F28, *leu*A6, *pyr*A1, *csp*A1) a derivative of *A. niger* N400 (CBS 120.49) from the laboratory collection was used for transformation and was grown in minimal medium (9), pH 6.0, containing 1% (mass/volume) fructose, 0.1% (mass/volume) yeast extract, 0.02% (mass/volume) leucine, 0.02% (mass/volume) uridine, and a trace element solution according to Ref. 10. *Escherichia coli* DH5αF′[F′/*end*A1 *hsdR*17 ($r_K^-m_K^+$) *supE*44 *thi*-1 *rec*A1 *gyrA* (Nal^r) *rel*A1 Δ(*lacIZYA-argF)U169 deoR* (*ϕ80dlac*Δ*(1acZ)M15*] (11) was used for cloning throughout. λ phages were propagated in *E. coli* LE392 [F′ *e*14$^-$ (*Mcr*A$^-$) *hsdR*514 ($r_K^-m_K^+$) *supE*44 *supF*58 Δ(*lac*IZY)6 *galK*2 *galT*22 *metB*1 *trpR*55] (12). *E. coli* BB4 [F′ *lac*I^qZΔM15 *proAB* Tn10 (tet^r)] and

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y15627.

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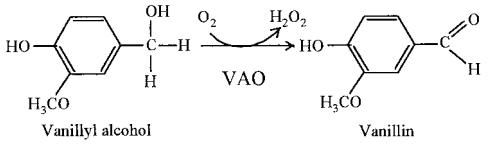

EQUATION 1

E. coli SOLR (el4-*(mcrA) Δ(mcrCB-hsdSMR-mrr)171 sbcC recB recJ uvrC umuC*::Tn5(kan^r) *lac gyrA96 relA1 thi-1 endA1* λ^R [F′ *lacI*^{q}*ZΔM15 proAB* Tn10 (tet^r)] *Su*$^-$) were used for cDNA cloning according to the suppliers recommendations (Stratagene Cloning Systems, La Jolla, CA). *E. coli* TG2 [Δ(*lac-pro*) *thi supE recA* [*Res*$^-$ *Mod*$^-$ (k)] F′(traD36 proA$^+$*B*$^+$ *lacI*^{q}*ZΔM15*)] (13) was used for expression of vanillyl-alcohol oxidase. *E. coli* strains were grown in Luria-Bertani medium supplemented with the appropriate antibiotic with the exception of *E. coli* LE392 which was grown in NZYCM medium. Media were solidified using 1.5% (mass/volume) bacto-agar. Top agar was prepared using NZYCM medium solidified with 0.7% agarose (electrophoresis grade).

Preparation of Cell Extracts—Cell extracts were prepared by sonication as described (6).

Enzyme Activity—Vanillyl-alcohol oxidase activity was determined at 25 °C in 50 mM potassium phosphate, 1 mM vanillyl alcohol, pH 7.5, by measuring the production of vanillin at 340 nm (ϵ_{340} vanillin = 14 mM^{-1} cm^{-1}).

Protein Sequence Analysis—Protein sequence analysis of vanillyl-alcohol oxidase was performed by Edman degradation at Eurosequence, Groningen, The Netherlands.

Manipulation of DNA—Isolation of phage and plasmid DNA and other molecular manipulations were carried out essentially as described (12). *A. niger* and *P. simplicissimum* chromosomal DNA was isolated according to de Graaff *et al*. (14). Restriction enzymes were used as recommended by the supplier (Life Technologies, Inc., Gaithersburg, MD).

The nucleotide sequence was determined using either the Cy5™ AutoCycle™ Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden) or the Cy5™-dATP Labeling Mix (Pharmacia Biotech). The reactions were analyzed with an ALFred™ DNA Sequencer. Computer analysis was done using the program DNAstar (Madison, WI).

Isolation of RNA—Total RNA was isolated by grinding frozen mycelium in liquid nitrogen-cooled shake flasks and grinding balls using a micro dismembrator (Braun Melsungen AG, Melsungen, Germany). Ground mycelium was extracted with TRIzol™ Reagent according to the supplier (Life Technologies, Inc.). Isolation of poly(A) tail mRNA was performed with oligo(dT)-cellulose obtained from Stratagene.

Construction and Screening of the cDNA Library—Using 5 μg of poly(A) tail mRNA, isolated from *P. simplicissimum* grown for 54 h on 0.1% (mass/volume) veratryl alcohol as the sole source of carbon (see "Results"), a cDNA library was constructed with a ZAP-cDNA Synthesis Kit using Uni-ZAP XR Vector Arms and a ZAP-cDNA Gigapack Gold III packaging extract (Stratagene). All procedures were carried out as described in the manual supplied with the cDNA Kit. The cDNA library was screened with purified antibodies raised against vanillyl alcohol oxidase, detection limit less than 10 pg (15), as described in the *pico*Blue™ Immunoscreening Kit instruction manual (Stratagene) using *E. coli* BB4 as a host. After a second immunoscreening *in vivo* excision of the pBlueScript phagemid from the Uni-ZAP XR Vector was performed using the ExAssist Helperphage with the *E. coli* SOLR strain according to the Single-Clone Excision Protocol (Stratagene).

Construction and Screening of the Genomic Library—P. simplicissimum chromosomal DNA was partially digested with *Sau*3AI and size-fractionated by agarose gel electrophoresis. 10–18-kilobase pair fragments were recovered from the gel by electroelution and cloned into the *Bam*HI sites of λ_{EMBL3} vector arms (Stratagene). Following packaging, the phages were used to infect *E. coli* LE392 and plated. After overnight growth phages were recovered from the top agar by extraction with SM buffer (12) yielding the primary library. The library was amplified by replating an aliquot of the recovered phages with *E. coli* LE392.

For screening of the amplified library dilutions were prepared yielding 7000–8000 plaques per plate. Four plates of phage were transferred in duplicate to Hybond N+ membranes (Amersham International plc, Little Chalfont, Buckinghamshire, UK) and processed as recommended by the supplier. Prehybridization and hybridization were carried out in 6× SSC, 5× Denhardt's solution, 0.5% (mass/volume) SDS at 65 °C. As a probe a 1215-bp[1] *Xba*I/*Bam*HI fragment of the *vao*A-cDNA was used (*Xba*I cuts at position 451 and *Bam*HI cuts at position 1923 in the genomic sequence as shown in Fig. 1). The fragment was labeled with [^{32}P]dATP as described previously (16). Washing steps were carried out for 30 min at 65 °C in the following solutions: twice in 2× SSC, 0.5% (mass/volume) SDS, and once in 0.5× SSC, 0.5% (mass/volume) SDS. For secondary screening identical conditions were applied with the exception that phage dilutions were used yielding 200–300 plaques per plate allowing the selection of individual plaques. Phages were propagated in *E. coli* LE392 and phage DNA isolated as described (12).

Transformation of A. niger—A. niger NW156 was transformed as described (17), using 1 μg of the selection plasmid pGW635 and 20 μg of cotransforming pIM3971. The copy number of the *P. simplicissimum vao*A gene in *A. niger* NW156-T10 was determined by Southern blot analysis. 5 μg of *Sal*I-digested *P. simplicissimum* chromosomal DNA and undiluted (5 μg) and serially diluted *Sal*I-digested *A. niger* NW156-T10 chromosomal DNA were separated by agarose gel electrophoresis, blotted, and subsequently hydridized with [^{32}P]dATP-labeled *vao*A-cDNA.

Expression Studies in P. simplicissimum and A. niger—Expression studies were carried out via transfer experiments. *P. simplicissimum* was precultured in medium described above using fructose (1%) as a carbon source. *A. niger* NW156-T10 was pregrown on complete medium (9) with 1% fructose as a carbon source. After 16 or 30 h of growth for *A. niger* and *P. simplicissimum*, respectively, mycelium was harvested and transferred to fresh media supplemented with carbon sources as described under "Results." At regular intervals mycelia were harvested and processed for RNA extraction or preparation of cell extract as described above.

Northern and Southern Analysis—Northern blots and Southern blots were carried out as described previously (12). [^{32}P]dATP-labeled *vao*A-cDNA was used as a probe (see above). The membranes were stripped according to the instructions of the manufacturer and rehybridized with a 900-bp *Eco*RI fragment encoding part of the 3′ end of the 28 S rDNA of *Agaricus bisporus* (18) to provide for a loading control.

Western Analysis—Western blot analysis was done as described previously (6) using the same antibodies as used for the cDNA library screening. Bound antibodies were detected by an alkaline phosphatase based immunoassay.

UV Visualization of Vanillyl-Alcohol Oxidase—Vanillyl-alcohol oxidase was visualized after SDS-PAGE as described (6).

PCR Mutagenesis to Introduce a Shine-Dalgarno Sequence—To enhance the expression of the *vao*A-cDNA a consensus Shine-Dalgarno sequence was introduced via PCR mutagenesis. This was done using the cDNA harboring plasmid pIM3970, taking advantage of the *Xba*I site at position 451 (numbering according to Fig. 1) and the *Kpn*I site from the polylinker of the vector pBlueScript SK downstream of the *vao*A-cDNA. Two oligonucleotides were used, primer 1, a 51-mer with the following sequence: 5′ GCGGACGTCGTTTA*AGAAGGAG*ATATACAT**ATGTCCAAGACACAGGAATTC** 3′, and primer 2, a 17-mer with the sequence 5′ CGAAGATTGTTCGCCTC 3′. Primer 1 is a mutagenic oligonucleotide. The sequence shown in boldface is identical to the N-terminal coding sequence, and the italicized sequence represents the Shine-Dalgarno sequence. Primer 2 is complementary to the sequence from positions 587 to 603 in Fig. 1 downstream of the *Xba*I site. PCR was performed in a Biometrica thermocycler in a 25-μl reaction volume containing PCR buffer (Pharmacia Biotech), 1.25 mM dNTP (each), 1 ng of *vao*A-cDNA, 100 pmol of each oligonucleotide, and 0.5 units of *Taq* polymerase (Pharmacia) with the following regime: 5 min at 95 °C; 30 cycles: 1 min at 95 °C, 1 min at 43 °C, and 1 min at 30 s 72 °C; 10 min at 72 °C.

The PCR fragment generated with primers 1 and 2 was cloned into pGEM-T (Promega Corp., Madison, WI) and sequenced to check the orientation and to check for undesired mutations. Next, the fragment in the right orientation with respect to the polylinker-encoded *Pst*I site was excised from pGEM-T by *Pst*I/*Xba*I digestion and ligated into *Pst*I/*Xba*I-digested pEMBL19. The gene was completed by cloning the *Xba*I/*Kpn*I fragment isolated from pIM3970 into the *Xba*I/*Kpn*I-digested previous pEMBL construct yielding plasmid pIM3972. The orientation of the gene is such that transcription takes place from the vector-encoded *lac* promoter.

Purification of Vanillyl-Alcohol Oxidase from E. coli TG2—E. coli TG2 cells carrying plasmid pIM3972 were grown batchwise in 500 ml of

[1] The abbreviations used are: bp, base pair(s); PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; *vao*A, vanillyl-alcohol oxidase encoding gene.

LB medium supplemented with 80 μg/ml ampicillin and 0.1 mM isopropyl-1-thio-β-D-galactopyranoside in 2-liter flasks in a rotary shaker set at 250 rpm at 37 °C. From 5-liter batch cultures cells were harvested by centrifugation and resuspended in 55 ml of 50 mM potassium phosphate buffer, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM $MgSO_4$, 10 mg of DNase I, pH 7.0. Cells were disrupted by passing three times through a precooled French pressure cell press operated at 10,000 p.s.i. Following centrifugation for 15 min at 15,000 × *g* to remove cellular debris, the supernatant was made 0.5% in protamine sulfate from a 2% mass/volume stock solution. Subsequently, the protamine sulfate aggregates were precipitated by centrifugation for 15 min at 15,000 × *g* followed by adjustment of the supernatant to 25% ammonium sulfate saturation. After centrifugation for 15 min at 15,000 × *g*, the supernatant was adjusted to 65% ammonium sulfate saturation. The precipitate was collected by centrifugation at identical settings as before. The pellet was dissolved in 50 mM potassium phosphate buffer, 0.5 mM EDTA, 0.5 M ammonium sulfate, pH 7.0, and loaded onto a phenyl-Sepharose column (30 × 2.6 cm) pre-equilibrated in the same buffer. The enzyme was eluted with a linear descending gradient from 0.5 to 0 M ammonium sulfate in the same buffer. Fractions were assayed for vanillyl-alcohol oxidase activity, pooled, and dialyzed against 25 mM potassium phosphate, pH 7.0. Next the dialysate was loaded onto a hydroxyapatite column (30 × 2.6 cm) pre-equilibrated with 25 mM potassium phosphate, pH 7.0. After washing the column with 3 volumes of starting buffer, the enzyme was eluted with a linear gradient of 25–300 mM potassium phosphate, pH 7.0. Fractions containing vanillyl-alcohol oxidase were pooled and concentrated in an Amicon ultrafiltration unit equipped with a YM-30 membrane.

RESULTS

Construction of the cDNA Library, Selection, and Sequence of the vaoA-cDNA—To ensure high abundance of the vanillyl-alcohol oxidase mRNA, total RNA was isolated from *P. simplicissimum* after 54 h of growth on minimal medium containing veratryl alcohol when vanillyl-alcohol oxidase activity was at 75% of its maximum (not shown). The primary library of $7 \cdot 10^4$ plaque-forming units was amplified to a titer of $1.4 \cdot 10^9$ plaque-forming units/ml. The first screening of the amplified library revealed that approximately 4.5% of the phages reacted with the vanillyl-alcohol oxidase-specific antibodies. From the second screening five positive phages were selected, and the phagemid was excised. Restriction enzyme analysis showed that the five phagemids contained identical inserts. From two such phagemids the nucleotide sequence of the insert was determined over both strands by sequencing subclones and by the use of specific oligonucleotides. The *vao*A-cDNA sequence and the derived amino acid sequence are presented in Fig. 1 together with the complete gene (see below). The *vao*A-cDNA nucleotide sequence revealed an open reading frame of 1680 bp encoding a protein of 560 amino acids with a deduced mass of 62,915 Da excluding FAD. Amino acid sequences obtained by automated Edman degradation of the N terminus of purified vanillyl-alcohol oxidase and of a purified internal peptide obtained by tryptic digestion of the enzyme were both identified in the deduced primary amino acid sequence (*bold type* in Fig. 1). The nucleotide sequence revealed that the first five amino acids were missing from the N-terminal peptide sequence suggesting some limited proteolytic processing of the enzyme in *P. simplicissimum*.

Cloning and Sequencing of the vaoA Gene—By using methods and conditions described under "Experimental Procedures" a genomic library of *P. simplicissimum* was constructed and subsequently screened with the [^{32}P]dATP-labeled *vao*A-cDNA as a probe. Two rounds of screening resulted in four positive phages that were characterized by restriction enzyme analysis and Southern blot analysis (results not shown). It was concluded that the entire gene should be located on an 8-kilobase pair *Sal*I fragment which was subsequently subcloned into *Sal*I-digested pUC18 yielding pIM3971. pIM3971 was used for sequencing either using specific oligomers based on the *vao*A-cDNA sequence and on sequences from the gene or by subclones generated. Fig. 1 shows the complete *vao*A gene, sequenced over both strands, including 582-bp promoter sequence and 293 bp of downstream sequence.

Comparison of the cDNA sequence with the genomic sequence revealed that the coding region is interrupted by five introns. The intron sequences follow the rules for filamentous fungi as proposed earlier (19): they are short, 61, 60, 70, 52, and 75 bp for introns i, ii, iii, iv and v, respectively, and the introns have consensus 5′ and 3′ splice sites, GTPuNGPy and PyAG, respectively, and lariat sequences, NNCTPuAPy (where Pu indicates purine and Py indicates pyrimidine), with only slight deviations.

The promoter region was analyzed for the presence of sequences involved in transcription regulation. The sequences 5′ GATA 3′ and 5′ GCCARG 3′ involved in nitrogen (20) and pH regulation (21), respectively, were not present. The context independent CreA binding site of *A. nidulans* 5′ GYGGGG 3′ (22) which is probably also recognized in *Penicillium* (23) was found once (position −557 to −552 in Fig. 1). The 5′ CAAT 3′ sequence, shown to be involved in transcription activation in *Saccharomyces* and other fungi (24), was found at positions −38, −61, −112, and −373 (*lowercase italic letters*). No TATA box was found in the sequence immediately upstream of the start codon. However, CT-rich sequences thought to direct transcription initiation (19) were present.

Vanillyl-Alcohol Oxidase Induction in P. simplicissimum and A. niger—Earlier studies have shown that *vao*A expression is gratuitously induced in *P. simplicissimum* when grown on veratryl alcohol (1) or anisyl alcohol (6). We have readdressed this issue in a transfer experiment in which veratryl alcohol was used as the sole source of carbon. Analysis was carried out by Northern and Western blotting and by vanillyl-alcohol oxidase activity determinations. In Fig. 2 the time course of induction is presented. *Panel A* shows the Northern blot probed with ^{32}P-labeled *vao*A-cDNA. In *panels B* and *C* the Western blot probed with the vanillyl-alcohol oxidase antibodies and the relative vanillyl-alcohol oxidase activity are presented, respectively. The results clearly show that at the start of the transfer no *vao*A-mRNA or vanillyl-alcohol oxidase enzyme is present. The vanillyl-alcohol oxidase activity and enzyme concentration follow the *vao*A-mRNA concentration with a lag, *i.e.* while the mRNA is maximal at 36 h the maximal activity is observed between 48 and 72 h. Furthermore, when *P. simplicissimum* was grown on the combination of 0.1% (mass/volume) veratryl or 0.1% (mass/volume) anisyl alcohol and 1% (mass/volume) glucose no vanillyl-alcohol oxidase was produced (results not shown) indicating that the gene is carbon catabolite-repressed. This was also suggested by the presence of the context independent CreA binding site in the promoter region.

A. niger NW156-T10, a pIM3971 multicopy transformant harboring 25–30 *vao*A copies, was used to study vanillyl-alcohol oxidase expression. Southern analysis using the *vao*A-cDNA as a probe demonstrated that *A. niger* NW156 does not contain a *vao*A gene with sufficient homology with the *P. simplicissimum vao*A gene to be detected under the conditions applied. In transfer experiments with *A. niger* NW156-T10 the inducing properties of veratryl alcohol, anisyl alcohol, vanillyl alcohol, veratric acid, vanillic acid, and 4-hydroxybenzoic acid were assessed 3 h after transfer (see Table I and Fig. 3). The highest level of vanillyl-alcohol oxidase activity was obtained with veratryl and anisyl alcohol, whereas no activity was seen when *A. niger* NW156-T10 was grown on fructose and when an untransformed *A. niger* strain was transferred to veratryl or anisyl alcohol (not shown). Both 4-methoxybenzyl alcohols were used alone and in combination with fructose to study the

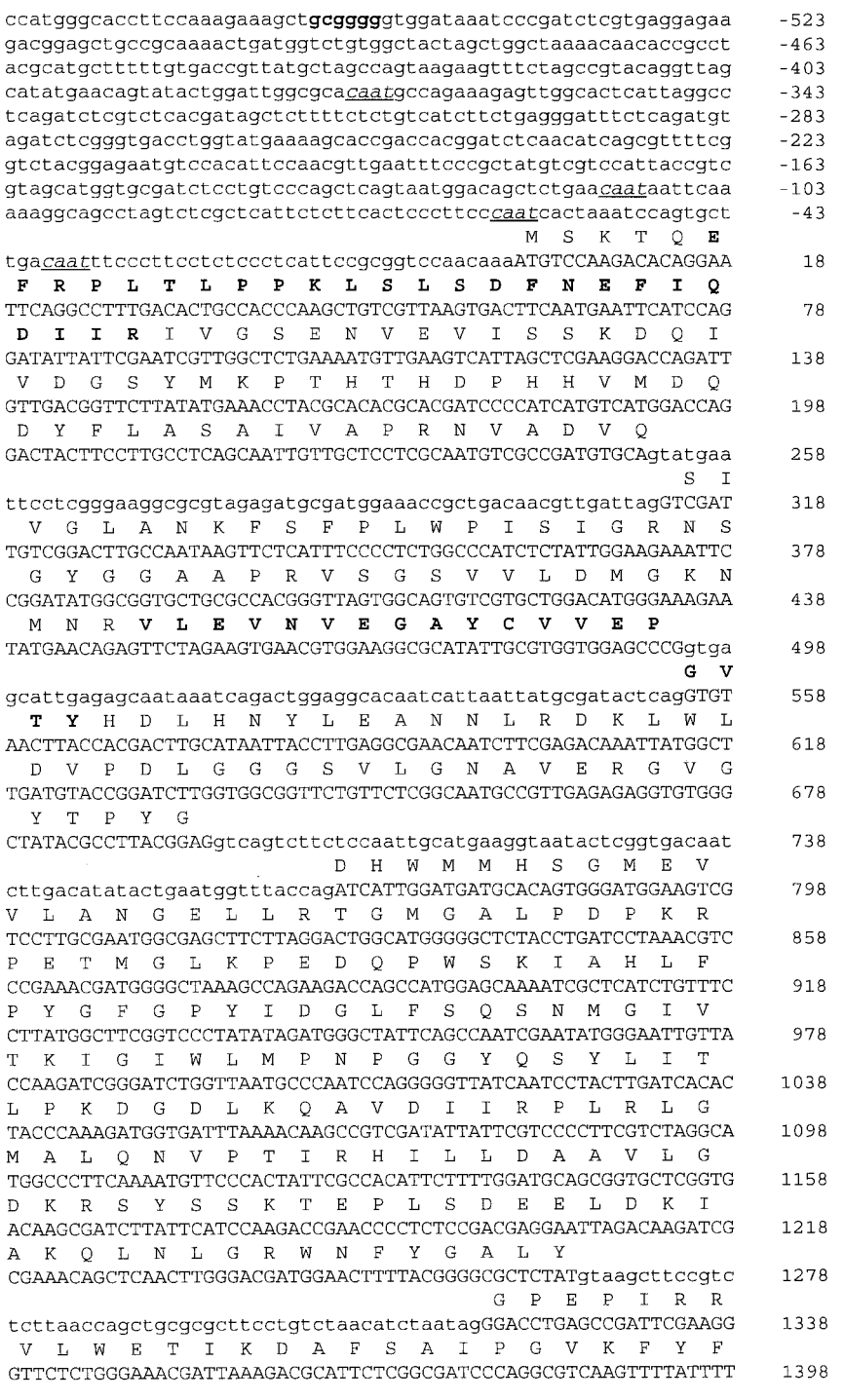

FIG. 1. **Nucleotide sequence and deduced amino acid sequence of the *vao*A gene and *vao*A-cDNA of *P. simplicissimum* encoding vanillyl-alcohol oxidase.** Coding sequence is shown in *capital letters*. Introns are indicated by *lowercase letters*. Promoter sequence is shown in *lowercase letters with negative numbering*. The downstream sequence of the gene is also represented by *lowercase letters*. The deduced amino acid sequence is indicated *above* the coding sequence. Amino acids shown in *boldface* were obtained by automated peptide sequencing via Edman degradation. The context independent CreA binding site is shown in *boldface lowercase letters*. The transcription activation sequence CAAT is indicated with *lowercase italic letters*. Polyadenylation was found at positions indicated with an *asterisk above* the nucleotide.

time course of vanillyl-alcohol oxidase induction (results not shown). With anisyl alcohol already after 3 h strong induction was observed both by Northern and Western analysis, whereas after 6 h the induction decreased. When fructose was included the induction was weaker both at 3 and 6 h. With veratryl alcohol induction was retarded when compared with anisyl alcohol. Fructose alone or a mixture of veratryl alcohol and fructose resulted in no detectable *vao*A-mRNA, and no vanillyl-alcohol oxidase was found by Western analysis.

Expression of vao-cDNA in E. coli TG2 and Purification of Recombinant Vanillyl-Alcohol Oxidase—Although vanillyl-alcohol oxidase could easily be detected with the vanillyl-alcohol oxidase-specific antibodies during screening of the cDNA library, the expression of the gene was quite low in *E. coli* (less than 0.5% of total protein, based on the specific activity of vanillyl-alcohol oxidase). In addition to the codon usage (see "Discussion"), the apparent lack of a good ribosome binding site may be the cause of this. To enhance the expression level of vanillyl-alcohol oxidase, a consensus Shine-Dalgarno sequence was introduced via PCR mutagenesis as described under "Experimental Procedures." Cell extracts of *E. coli* TG2 harboring pIM3972 showed a 7-fold increase of expression of vanillyl-alcohol oxidase when compared with *E. coli* TG2 transformed with pIM3970.

Recombinant vanillyl-alcohol oxidase was purified from *E. coli* TG2 harboring pIM3972 in a two-column procedure (Table II). The recombinant enzyme migrated as a single band in SDS-PAGE (Fig. 4) and was identical with vanillyl-alcohol

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 P  E  D  T  P  E  N  S  V  L  R  V  R  D  K  T  M  Q  G  I
CCGGAGGACACTCCTGAAAACTCCGTTCTCCGCGTGCGTGATAAGACTATGCAAGGCATT    1458
 P  T  Y  D  E  L  K  W  I  D  W  L  P  N  G  A  H  L  F  F
CCAACTTACGACGAGCTAAAGTGGATCGATTGGCTCCCTAATGGTGCGCATCTGTTCTTC    1518
 S  P  I  A  K  V  S  G  E  D  A  M  M  Q  Y  A  V  T  K  K
TCTCCTATTGCGAAGGTATCTGGTGAAGATGCAATGATGCAATACGCAGTCACCAAGAAA    1578
 R  C  Q  E  A  G  L  D  F  I  G  T  F  T  V  G  M  R  E  M
AGGTGTCAGGAGGCTGGGTTAGATTTTATCGGCACTTTCACAGTCGGTATGAGAGAGATG    1638
Cgtgcgtcaatccccagacccctcagaaacattccttcccttgctcgggatagctaatct    1698
                H  H  I  V  C  I  V  F  N  K  K  D  L  I  Q
ccgtgttattgtacagATCATATCGTTTGTATTGTGTTCAACAAGAAGGACCTAATACAA    1758
 K  R  K  V  Q  W  L  M  R  T  L  I  D  D  C  A  A  N  G  W
AAGAGAAAAGTACAGTGGCTGATGAGAACCCTTATTGATGACTGTGCTGCAAATGGATGG    1818
 G  E  Y  R  T  H  L  A  F  M  D  Q  I  M  E  T  Y  N  W  N
GGCGAATATCGAACCCATCTGGCCTTCATGGACCAAATTATGGAAACCTACAACTGGAAC    1878
 N  S  S  F  L  R  F  N  E  V  L  K  N  A  V  D  P  N  G  I
AACAGCAGCTTCCTAAGGTTCAATGAGGTCCTCAAGAATGCGGTGGATCCTAATGGCATC    1938
 I  A  P  G  K  S  G  V  W  P  S  Q  Y  S  H  V  T  W  K  L
ATTGCCCCGGGAAAGTCTGGTGTTTGGCCGAGTCAATACAGTCATGTTACTTGGAAACTG    1998
 #
taagcccgatgggtccaagagcttttatttctgttaagaaaatcgatttatcttttcatt    2058
      *               *
tatccagatttggagtgaaagttcccgaattcgagaagattgtctcgcaaattaccggct    2118
aatcaacgtgaaccagcgacgtatcaatggcacaactacgaaaagtattacttacccata    2178
ttacctataactcggtctcatcctgtggcgtaaacgacgatcaaacagcagccgctatgt    2238
gtgacacttcatgaccttccgaattaaaaattcactcgacccacaaggaagtgttag       2294
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FIG. 1—*continued*

oxidase from *P. simplicissimum* (1) in all aspects tested: spectral properties (250–520 nm), steady state kinetic parameters for vanillyl alcohol, and the association into octamers.

DISCUSSION

In this paper we have described the cloning and sequencing of the gene encoding vanillyl-alcohol oxidase from *P. simplicissimum,* the first 8α-(N^3-histidyl)-FAD containing enzyme of known three-dimensional structure (7). The gene is strongly induced in *P. simplicissimum* when the fungus is grown on 4-methoxybenzyl alcohols (6). This high amount of enzyme is reflected in the abundance of *vao*A-cDNA clones (4.5%) present in the cDNA library constructed with mRNA isolated from veratryl alcohol-grown *P. simplicissimum*. Five independent cDNA clones appeared identical at the physical map level. Two of these clones were fully sequenced and shown to encode the complete vanillyl-alcohol oxidase. This indicates that the majority of cDNA clones are full-length clones.

The *vao*-cDNA nucleotide sequence encodes an open reading frame of 1680 bp corresponding to a 560-amino acid protein with a deduced mass of 62,915 Da excluding the covalently bound FAD which is slightly lower than the value of 65 kDa as estimated previously from SDS-PAGE (1). Apart from the first five N-terminal amino acids that were not present in the purified enzyme, the sequence of the deduced amino acids was consistent with the N-terminal sequence obtained from the purified protein. The identity of the cDNA was further confirmed by the amino acid sequence of a purified vanillyl-alcohol oxidase peptide (residues 130–148). Conclusive evidence was obtained by the demonstration of vanillyl-alcohol oxidase activity in *E. coli* cells transformed with the *vao*A-cDNA harboring plasmid pIM3970.

Comparison of the deduced amino acid sequence for vanillyl-alcohol oxidase using available data bases revealed 31% sequence identity with the flavoprotein subunit of the bacterial flavocytochrome *p*-cresol methylhydroxylase (25) (Fig. 5). This $\alpha 2\beta 2$ heterotetramer catalyzes the oxidation of *p*-cresol first to *p*-hydroxybenzyl alcohol and then to *p*-hydroxybenzaldehyde (26). These consecutive reactions are also catalyzed by vanillyl-alcohol oxidase, although at a lower rate (8). Vanillyl-alcohol oxidase and *p*-cresol methylhydroxylase both contain a covalently bound FAD, but the mode of covalent linkage is not conserved. In vanillyl-alcohol oxidase, the 8α-carbon of the flavin is bound to the N-3 atom of His-422 (7), whereas in *p*-cresol methylhydroxylase, the 8α-carbon of the flavin is bound to the phenolic oxygen of Tyr-384 (25). Furthermore, as can be seen from Fig. 5, Tyr-384 of *p*-cresol methylhydroxylase is shifted 8 residues toward the N terminus compared with His-422 of vanillyl-alcohol oxidase.

The crystal structure of vanillyl-alcohol oxidase shows that each monomer is composed of two domains (7). The larger domain (residues 6–270 and 500–560) binds the ADP part of the FAD, whereas the cap domain (residues 271–499) covers the isoalloxazine ring. The folding topology of the vanillyl-alcohol oxidase subunit closely resembles that of the flavoprotein subunit of *p*-cresol methylhydroxylase (27, 28). From this and the data presented in Fig. 5, it is clear that the most conserved parts of the sequence (101–147, 178–219, and 245–271) concern residues that are located in the FAD binding domain. Several active site residues are also conserved. These include Tyr-108, Tyr-503, and Arg-504 that are involved in binding the phenolic moiety of the substrate. In vanillyl-alcohol oxidase, these residues facilitate substrate deprotonation upon binding (3). Asp-170, which is thought to play a crucial role in the catalytic mechanism of vanillyl-alcohol oxidase (7), is not conserved (Fig. 5). This might explain the different reactivities of both enzymes toward *p*-cresol (8). However, a detailed comparison of the active sites of vanillyl-alcohol oxidase and *p*-cresol methylhydroxylase requires the completion of the crystallographic refinement of the *p*-cresol methylhydroxylase structure. Besides the 31% sequence identity with *p*-cresol methylhydroxylase, no strong sequence identity was found between vanillyl-alcohol oxidase and other enzymes. However, from the crystal structure determination (7), it has become apparent that the folding topology of the FAD-binding domain of vanillyl-alcohol oxidase resembles that of MurB, a flavoenzyme involved in the biosynthesis of the bacterial cell wall (29). Moreover, it has been suggested that this unusual FAD-binding fold is shared by other flavoprotein oxidoreductases (30, 31).

In *P. simplicissimum* the *vao*A gene is induced by a limited amount of aromatic compounds (6). Apart from 4-(methoxymethyl)phenol, which may represent the natural substrate, the non-vanillyl-alcohol oxidase substrates anisyl alcohol and veratryl alcohol are potent inducers. Also in *A. niger* NW156-T10,

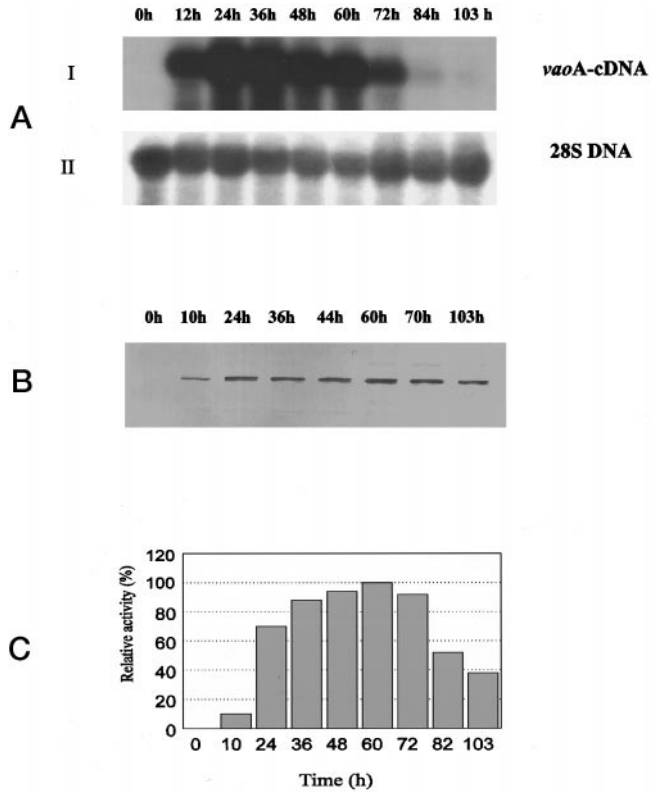

FIG. 2. **Time course of induction of *P. simplicissimum vao*A transcription and translation.** *P. simplicissimum* was pregrown on fructose and transferred to medium containing veratryl alcohol (see "Experimental Procedures"). Samples were taken at times indicated. *Panel A*, Northern blot probed with ^{32}P-labeled *vao*A-cDNA (*I*) and reprobed with ^{32}P-labeled *A. bisporus* 28 S rDNA as a loading control (*II*). *Panel B*, Western blot screened with antibodies raised against vanillyl-alcohol oxidase. *Panel C*, vanillyl-alcohol oxidase relative activity profile.

TABLE I
Specific activity of vanillyl-alcohol oxidase from the multicopy vaoA transformant Aspergillus niger NW156-T10

A. niger NW156-T10 was pregrown on fructose and transferred to different substrates. Cell extract was prepared by sonication as described previously (6). Untransformed *A. niger* NW156 showed no vanillyl-alcohol oxidase activity when transferred to veratryl alcohol and anisyl alcohol.

Substrate	Specific activity
	units/mg
Fructose	0.000
Anisyl alcohol	0.484
Ferulic acid	0.079
4-Hydroxybenzoic acid	0.124
Vanillic acid	0.175
Vanillyl alcohol	0.136
Veratric acid	0.209
Veratryl alcohol	0.346

transformed with 25–30 copies of the *vao*A gene, strong expression of the gene was observed with these methoxybenzyl alcohols. With 4-hydroxybenzoic acid, ferulic acid, vanillic acid, and vanillyl alcohol vanillyl-alcohol oxidase was detected as well. This indicates that the *vao*A gene in *A. niger* NW156-T10 is rendered under the control of at least one regulator involved in regulation of genes involved in the metabolism of aromatic compounds. Furthermore, the *vao*A gene is both in *A. niger* NW156-T10 and *P. simplicissimum* under the control of carbon catabolite repression. Since the *vao*A gene is expressed from its own promoter, this means that both in *A. niger* NW156-T10 and *P. simplicissimum* similar regulation mechanisms must be

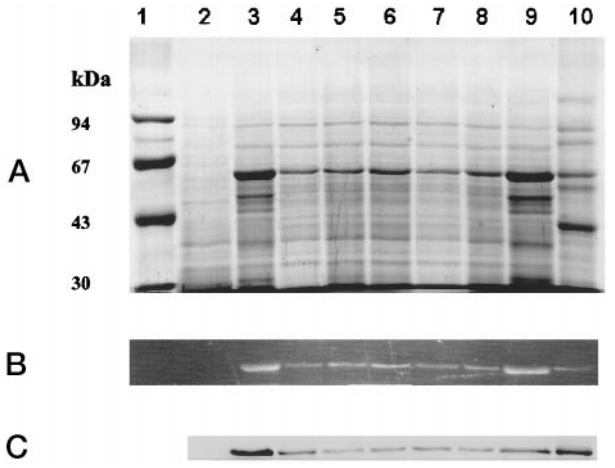

FIG. 3. **Effect of various carbon sources on *P. simplicissimum vao*A expression in *A. niger* NW156-T10.** *A. niger* NW156-T10 transformed with 25–30 copies of the *P. simplicissimum vao*A gene pregrown on fructose was transferred to the following substrates: fructose (*lane 2*), anisyl alcohol (*lane 3*), ferulic acid (*lane 4*), 4-hydroxybenzoic acid (*lane 5*), vanillic acid (*lane 6*), vanillyl alcohol (*lane 7*), veratric acid (*lane 8*), and veratryl alcohol (*lane 9*) and analyzed 3 hours after transfer. Extracts were prepared by sonication. *P. simplicissimum* grown on veratryl alcohol (*lane 10*) served as a control. *Lane 1*, marker. *Panel A*, 10% SDS-PAGE gel stained with Coomassie Brilliant Blue. *Panel B*, UV-visualized vanillyl-alcohol oxidase. *Panel C*, Western blot screened with vanillyl-alcohol oxidase-specific antibodies.

TABLE II
Purification of recombinant vanillyl-alcohol oxidase from E. coli TG2 transformed with pIM3972 harboring the P. simplicissimum vaoA-cDNA

Step	Protein	Activity	Specific activity	Yield
	mg	*units*	*units/mg*	*%*
Cell extract	2640	46	1.7×10^{-2}	100
Protamine sulfate	1648	42	2.5×10^{-2}	91
Ammonium sulfate treatment	801	37	4.6×10^{-2}	80
Phenyl-Sepharose chromatography	88	38	0.4	83
Hydroxyapatite chromatography	24	35	1.5	76

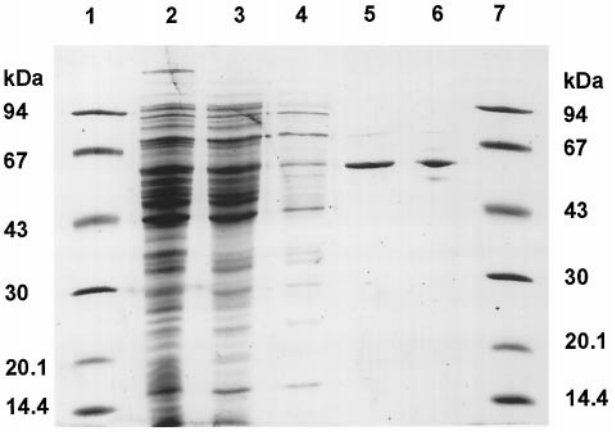

FIG. 4. **SDS-PAGE of purification steps of recombinant vanillyl-alcohol oxidase from *E. coli* TG2.** The Coomassie Brilliant Blue-stained 10% SDS-PAGE gel contains the following: *lanes 1* and *7*, marker S4; *lane 2*, cell extract; *lane 3*, 65% ammonium sulfate precipitate; *lane 4*, phenyl-Sepharose pool; *lane 5*, hydroxyapatite pool; *lane 6*, vanillyl-alcohol oxidase purified from *P. simplicissimum*.

operative. In both organisms a surprisingly high expression level of vanillyl-alcohol oxidase is observed in the presence of veratryl and anisyl alcohol. This may be explained by assuming that these compounds, or one of their metabolites, have a high affinity for the common aromatic pathway regulator(s), most likely repressor(s). The affinity must be much higher than the

```
VAO   MSKTQEFRPLTLPPKLSLSDFNEFIQDIIRIVGSENVEVISSKDQIVD-G  49
PCMH  MSEQNN---AVLPKGVTQGEFNKAVQKFRALLGDDNVLVES--DQLVPYN  45

VAO   SYMKPTHT--HDPHHVMDQDYFLASAIVAPRNVADVQSIVGLANKFSFPL  97
PCMH  KIMMPVENAAHAP----------SAAVTATTVEQVQGVVKICNEHKIPI  84

VAO   WPISIGRNSGYGGAAPRVSGSVVLDMGKNMNRVLEVNVEGAYCVVEPGVT 147
PCMH  WTISTGRNFGYGSAAPVQRGQVILDL-KKMNKIIKIDPEMCYALVEPGVT 133

VAO   YHDLHNYLEANNLRDKLWLDVPDLGGGSVLGNAVERGVGYTPYGDHWMMH 197
PCMH  FGQMYDYIQENNLPVMLSFSAPSAIAGPV-GNTMDRGVGYTPYGEHFMMQ 182

VAO   SGMEVVLANGELLRTGMGALPDPKRPETMGLKPEDQPWSKIAHLFPYGFG 247
PCMH  CGMEVVLANGDVYRTGMGGVPG----------SNTW----QIFKWGYG 216

VAO   PYIDGLFSQSNMGIVTKIGIWLMPNPGGYQSYLITLPKDGDLKQAVDIIR 297
PCMH  PTLDGMFTQANYGICTKMGFWLMPKQPVFKPFEVIFEDEADIVEIVDALR 266

VAO   PLRLGMALQNVPTIRHILLDAAVLGDKRS-YSSKTEPLSDEELDKIAKQL 346
PCMH  PLRMSNTIPNSVVIASTLWEAGSAHLTRAQYTTEPGHTPDSVIKQMQKDT 316

VAO   NLGRWNFYGALYGPEPIRRVLWETIKDAFSAIPGVKFYFPEDTPENSVLR 396
PCMH  GMGAWNLYAALYGTQEQVDVNWKIVTDVFKKLGKGRIVTQEEAGDTQPFK 366
                                      ↓
VAO   VRDKTMQGIPTYDELKWIDWLPNGAHLFFSPIAKVSGEDAMMQYAVTKKR 446
PCMH  YRAQLMSGVPNLQEFGLYNWRGGGGSMWFAPVSEARGSECKKQAAMAKRV 416
                      ↑
VAO   CQEAGLDFIGTFTVGMREMHHIVCIVFNKKDLIQKRKVQWLMRTLIDDCA 496
PCMH  LHKYGLDYVAEFIVAPRDMHHVIDVLYDRTNPEETKRADACFNELLDEFE 466

VAO   ANGWGEYRTHLAFMDQIMETYN-----WNNSSFLRFNEV---LKNAVDPN 538
PCMH  KEGYAVYRVNTRFQDRVAQSYGPVKRKWSMPSSVRWTRTTSSLRAARAST 516

VAO   GIIAPGKSGVWPSQYSHVTWKL 560
PCMH  SITISDADG-WLATG------- 530
```

FIG. 5. **Sequence alignment between vanillyl-alcohol oxidase and the flavoprotein subunit of *p*-cresol methylhydroxylase.** *Vertical lines* represent identical amino acids; *dots* indicate conserved residues. *Gaps* introduced for optimal alignment are indicated with *dashes*; residues forming the covalent bond with FAD are indicated with *arrows*, and active site residues are in *boldface* and are *underlined*.

affinity of 4-hydroxybenzoic acid, ferulic acid, and vanillyl alcohol or their metabolites. These latter compounds induce vanillyl-alcohol oxidase expression in *A. niger* NW156-T10 but not in *P. simplicissimum*. The discrepancy in the level of vanillyl-alcohol oxidase expression between *A. niger* NW156-T10 and *P. simplicissimum* during growth on 4-hydroxybenzoic acid, ferulic acid, and vanillyl alcohol may be accounted for by the following: (i) in *A. niger* NW156-T10 25–30 copies of the *vao*A gene are present *versus* 1 copy in *P. simplicissimum*, and (ii) in the present study *A. niger* NW156-T10 mycelia were harvested 3 or 6 h following transfer, whereas in the studies with *P. simplicissimum* mycelia were allowed to grow 2 days after transfer which may have caused degradation of vanillyl-alcohol oxidase due to toxic effects and/or the poor carbon sources these aromatic compounds represent (6).

Although expression of *vao*A-cDNA in *E. coli* TG2 cells was evident, since specific antibodies could be used to select the cDNA, the expression level was low. It was previously observed that *E. coli* TG2 cells are capable of producing relatively high amounts (>50 mg/liter of culture) of recombinant enzymes from multicopy plasmids like pBlueScript and pUC under the direction of the plasmid-encoded *lac* promoter (32, 33). Inspection of the *vao*A-cDNA sequence revealed two possible explanations for the low expression. The first reason may be the codon usage. Codons that are considered modulator codons in *E. coli*, suppressing high expression (34), occur with a relatively high frequency in the cDNA. The second reason may be the apparent absence of a good ribosome binding site. The

introduction of a consensus *E. coli* ribosome binding site at the correct distance from the start codon increased the expression level only 7-fold indicating that the low expression is related to the codon usage.

Finally, this study has clearly established that expression of the *vao*A gene in a prokaryotic or eukaryotic host results in active, fully covalently flavinylated enzyme. This suggests that the flavinylation is an autocatalytic process as shown for 6-hydroxy-D-nicotine oxidase (35, 36). However, for *p*-cresol methylhydroxylase it was shown that autocatalytic flavinylation only occurred after binding of the cytochrome subunit (28).

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